1 **Mitochondrial behaviour throughout the lytic cycle of *Toxoplasma gondii***

2

3

4 Jana Ovciarikova[1], Leandro Lemgruber[1], Krista L. Stilger[2], William J. Sullivan

5 Jr.[2,3] and Lilach Sheiner[1*]

6

7 [1]Wellcome Trust Centre for Molecular Parasitology, University of Glasgow, UK

8 [2]Department of Pharmacology and Toxicology, Indiana University School of

9 Medicine, Indianapolis, Indiana 46202, USA

10 [3]Department of Microbiology and Immunology, Indiana University School of

11 Medicine, Indianapolis, Indiana 46202, USA

12 [*]Corresponding author: lilach.sheiner@glasgow.ac.uk

13

17

18

19

Abstract

Mitochondria distribution in cells controls cellular physiology in health and

disease. Here we describe the mitochondrial morphology and positioning found

in the different stages of the lytic cycle of the eukaryotic single-cell parasite

Toxoplasma gondii. The lytic cycle, driven by the tachyzoite life stage, is

responsible for acute toxoplasmosis. It is known that whilst inside a host cell the

tachyzoite maintains its single mitochondrion at its periphery. We found that upon

parasite transition from the host cell to the extracellular matrix, mitochondrion

morphology radically changes, resulting in a reduction in peripheral proximity.

This change is reversible upon return to the host, indicating that an active

mechanism maintains the peripheral positioning found in the intracellular stages.

Comparison between the two states by electron microscopy identified regions of

coupling between the mitochondrion outer membrane and the parasite pellicle,

whose features suggest the presence of membrane contact sites, and whose

abundance changes during the transition between intra- and extra-cellular states.

These novel observations pave the way for future research to identify molecular

mechanisms involved in mitochondrial distribution in *Toxoplasma* and the

consequences of these mitochondrion changes on parasite physiology.

Introduction

The various functions of mitochondria are governed in part by positioning of the organelle at relevant cellular locations. The dynamic nature of mitochondrial behavior is indicated by the multiple morphologies seen in various cell types and under various growth conditions. For example, several mammalian cell-types show morphologically heterogeneous and unconnected mitochondria in a steady state[1]. On the other hand, mitochondria can assume a highly connected tubular structure, e.g. during the development of cardiomyocytes[2]; or a highly fragmented morphology as in the case of yeast sporulation[3]. In another example, the eukaryotic parasite *Trypanosoma brucei* presents a small, unbranched tube-like mitochondrion during the life-cycle stage when it is not involved in ATP production and a branched mitochondrion when the TCA cycle is active[4] [5] [6]. Little is known about morphological changes that may occur in the mitochondrion of the eukaryotic unicellular parasite *Toxoplasma gondii* (*T. gondii*). Examination of mitochondrial morphology in the rapidly replicating life stage (tachyzoite), when intracellular, shows a single mitochondrion which is predominantly found in a lasso shape that spans the parasite periphery[7,8]. This lasso shape is the main morphology described for the *Toxoplasma* mitochondrion in the current literature. Interestingly, during tachyzoite cell division the mother mitochondrion maintains close proximity to the mother-cell periphery and is excluded from the growing daughters until the late stages of cytokinesis[9].

T. gondii must reside within a nucleated host cell to grow and divide. The

63   tachyzoite stage will spend time in the extracellular environment after egress

64   from a host cell, when it seeks a new host cell to invade, and thus continues its

65   "lytic cycle". Recent observations suggest that extracellular tachyzoites are also

66   found free in the blood stream of infected mice[10]. The transition from intracellular

67   to extracellular conditions is accompanied by drastic changes in ion

68   concentration and nutrient availability. The ability to survive outside the host cell,

69   move around and then invade a new host cell is critical to the *T. gondii* lifestyle

70   and virulence.

71

72   As a first step to tease out a potential function-morphology correlation in the *T.*

73   *gondii* mitochondrion, we looked for any morphological changes that can be

74   observed during the lytic cycle. We found that extracellular parasites exhibit

75   drastic changes in mitochondrial morphology immediately after being released

76   from the host cells. These changes are characterized by detachment of the

77   mitochondrial tubule from the parasite periphery and its accumulation in

78   concentrated regions in the cell. These changes seem directional and reversible

79   upon host cell re-entry. Electron microscopy links these striking morphological

80   dynamics to a change in the abundance of long patches of high proximity

81   between the parasite's mitochondrion and the parasite's alveoli-sacs at its

82   pellicle. These observations pave the way for future studies of the molecular

83   mechanisms controlling apicomplexan mitochondrial behavior and how it

84   contributes to survival of parasites between intra- and extracellular states.

85

86   **Results**

87

88   **Morphological changes in the mitochondrion of extracellular tachyzoites**

89

90   Most previously available imaging of mitochondrial morphology and dynamics in

91   live *T. gondii* utilized a matrix marker whereby the leader sequence of

92   mitochondrial HSP60 is fused to the red fluorescent protein and the resulting

93   fusion is expressed from a heterologous promoter[9]. We generated a fluorescent

94   marker for the mitochondrial periphery via fusion of the TGME49_215430

95   encoded protein[11] to the yellow fluorescent protein (YFP) by endogenous

96   tagging. This protein was found in a search for *T. gondii* proteins that contain

97   each a single hydrophobic domain, and within this screen it was localized to the

98   mitochondrion (Sheiner and Soldati unpublished work). Homologs of this protein

99   are only found in organisms from the Alveolata group (that includes within it the

100   phylum Apicomplexa to which *T. gondii* belongs). No functional domains are

101   predicted; however a lipid attachment site is predicted at the N-terminus

102   (http://prosite.expasy.org/PS51257) that suggest potential attachment to the

103   mitochondrial membrane. TGME49_215430 endogenously tagged with YFP

104   (215430-YFP) co-localizes with the outer-mitochondrial membrane marker

105   Tom40 [12] as well as the signal obtained from Mitotracker® (Figure 1a,b). We

106   observed that imaging with this marker identifies mitochondrial structures that are

107   not labeled using the matrix marker (Figure 1c, arrowhead). It further labels a

5

108   continuous mitochondrial tubule, whereas the matrix signal is fragmented (Figure

109   1d, arrowheads). 215430-YFP is used throughout this report.

110   We revisited the previous observation on mitochondrial morphology in

111   intracellular parasites. First we validated the new marker by reproducing the

112   observations of the unique mitochondrial behavior in dividing tachyzoites[9] (Figure

113   S1a,b,c, Movie S1). Next, we scored morphologies in intracellular parasites. *T.*

114   *gondii* intracellular replication is asynchronous and different parasites are at

115   different stages of the cell cycle at a given time. Nevertheless, we observed that

116   an average of 94% of intracellular parasites possess the typical lasso shaped

117   mitochondria with peripheral localization (Figure 2), supporting earlier

118   observations that this is the predominant morphology maintained throughout the

119   intracellular cell cycle[7]. Live imaging show that the majority of parasites contain a

120   lasso shaped mitochondrion also just before egress (Movie S2). However, due to

121   the constant movement of the parasites within cells in the Z axis during imaging

122   we were unable to determine the mitochondrial morphology with accuracy in all

123   the parasites, making quantification difficult. In contrast, we found that

124   immediately after host cell egress, *T. gondii* mitochondria present an array of

125   morphologies (Figure 2a,b), only 35% of which retain the lasso shape (Figure

126   2c). We assigned these extracellular mitochondrial morphologies to three main

127   categories based on their shapes and termed them "lasso", "sperm-like" and

128   "collapsed" (Figure 2d). An average of 42% of freshly released parasites have

129   "sperm-like" mitochondria (Figure 2c), which consist of a round bundle of folded

130   mitochondrial tubule, with a part of the tubule remaining extended. 21% of

131   extracellular parasites show completely collapsed mitochondrial tubules (Figure

132   2c). Similar distribution of these three morphologies are also observed when

133   parasites egress into diluted medium that better mimics the low nutrient

134   environment encountered by the parasite in the extracellular matrix *in*

135   *vivo*[13](Figure 2c). Equally, similar distribution of these morphologies is observed

136   when parasites egress is induced via the calcium ionophore, ionomycin (Figure

137   2c), with a moderate elevation in the proportion of collapsed mitochondria.

138

139   Changes in mitochondrial morphology were observed in *T. gondii* previously, in

140   response to starvation and drug treatment. These studies describe mitochondrial

141   swelling and fragmentation observed after 6 or 8 hours of treatment[14] [15]. We

142   noticed occasional freshly egressed parasites showing fragmentation or swelling

143   similar to what was observed following starvation or drug treatment. In all cases,

144   unlike the lasso, sperm-like and collapsed forms, this was coupled to an overall

145   abnormal cell morphology documented by brightfield imaging (Figure S2a).

146   Comparison of these morphologies via super-resolution microscopy (3D-SIM)

147   using the peripheral marker 215430-YFP showed that swollen and fragmented

148   mitochondria have different structures to the packed folded tubule observed

149   immediately upon host-cell release (Figure S2b). Furthermore, extracellular

150   parasites with swollen and fragmented mitochondria stain with propidium-iodide

151   (PI), indicating cell death has commenced. Additionally, their mitochondria are

152   not labelled with Mitotracker[®], suggesting they are no longer active (Figure

153   S2c,d). Parasites with fragmented or swollen mitochondria were thus excluded

154   from our analysis.

155

156   **Mitochondrion morphology during extracellular gliding motility, host cell**

157   **invasion, and entry into a new host cell.**

158

159   During the lytic cycle (Figure 3a), extracellular tachyzoites use gliding motility to

160   find and enter their next host cell. We examined mitochondrial morphology in

161   moving parasites upon temperature-shift induction of gliding motility in fresh

162   mechanically-released parasites. The repertoire of morphologies in gliding

163   parasites is similar to that of the overall population of freshly released parasites

164   (33% lasso, 50% sperm-like and 17% collapsed) with no significant difference in

165   the frequencies of each shape (Figures 3b,c, Movie S3).

166   Host cell invasion is the next essential step of the *T. gondii* lytic cycle (Figure 3a).

167   Using the green/red assay ([16], Movie S4.1/2/3) to label invading parasites (Figure

168   3d), we scored parasites that were in the act of invasion. We found a similar

169   distribution of morphologies to those found in freshly egressed and in gliding

170   parasites (33% lasso, 55% sperm-like and 12% collapsed), albeit a moderate

171   increase in the frequency of sperm-like shaped mitochondria in the invading

172   population (Figure 3e).

173   Finally, parasites presenting the collapsed mitochondrial tubule morphology were

174   analyzed after entry into a new host cell. Following invasion, collapsed

175   mitochondria re-expand and re-establish the typical intracellular lasso shape

(Figure 4a, Movie S5). Among three independent live-imaging experiments the timing of re-expansion after invasion varied. However, cell division (as visualized by the appearance of daughter cells) occurred only after mitochondrial remodeling into a lasso shape (Figure 4a, Movie S5). In this context it is worth mentioning that when examining cultures of intracellular parasites, the 0.7% that contain a collapsed mitochondrion (Figure 2c) are always single parasites. We propose that these are parasites that recently invaded the host cell, and have not yet remodeled their mitochondrion to the lasso shape.

Interestingly, the transition from collapsed to lasso involves an intermediate sperm-like shape (Figure 4a arrowhead). Due to the rapid motility of extracellular parasites we were unable to capture the remodeling in the opposite direction (from lasso to collapsed) via time-lapse microscopy of freshly egressed parasites. However, we found that parasites that remain extracellular for 0, 6, 12 and 24 hours, and that do not possess swollen and fragmented mitochondria, show 37%, 9.3%, 3.7% and 1.5% lasso-shaped mitochondria respectively (Figure 4b). Within these time points the number of parasites with sperm-like mitochondria initially increased (from 45.5% to 66.2% at 6 hours) and then dropped (to 27.8% at 24 hours), while parasites with collapsed mitochondria gradually accumulated to become 70.7% at 24 hours (Figure 4b). This dynamic suggests a model whereby the sperm-like morphology is an intermediate form between lasso and collapsed. Analysis of the orientation of the tubule extension ("tail") of the sperm-like mitochondria showed that the tail is predominantly basal (Figure 4c), suggesting that the retraction from lasso to sperm-like is directional.

199

200   Collectively, these observations show that mitochondrial morphology in *T. gondii*

201   tachyzoites is dynamic during the lytic cycle. The mitochondrion responds to the

202   transitions between extracellular and intracellular stages in a controlled manner

203   that results in the remodeling into a lasso shape observed after host cell

204   invasion.

205

206   **Patches of tight mitochondrion-pellicle proximity are observed, and their**

207   **abundance correlates with mitochondrial remodeling**

208

209   We investigated the cellular structures linked to the peripheral positioning of the

210   mitochondrion in intracellular tachyzoites. The behavior of mitochondria in other

211   eukaryotic cells is controlled via several molecular mechanisms[17]. In many

212   systems, components of the cell cytoskeleton play a central role in this process.

213   We assessed mitochondrial morphology in response to treatment with the

214   microtubule destabilization agent Oryzalin. Fixed cell microscopy showed that

215   while this treatment results in irregular mitochondrion morphology, the results

216   were distinct from the above described morphologies in extracellular parasites

217   (Figure S3a). Moreover, live-imaging of tachyzoite cell division under Oryzalin

218   treatment shows that mitochondria enter the new daughter cells while still

219   forming lasso-like shapes (Figure S3b arrowheads, Movie S6).

220

221   Mitochondrial morphology is also shaped through its interactions with other

222   organelles. We investigated whether other organelles show behavior that may be

223   linked to the observed mitochondrial morphology changes upon the transition

224   from intracellular to extracellular stages. We analyzed the morphologies and

225   distribution of the three other organelles that occupy a large part of the tachyzoite

226   cell and that show association with the mitochondrion in intracellular parasites[7]:

227   the rhoptries, parasite-specific organelles that stretch from the apical tip to the

228   nucleus; the nucleus; and the endoplasmic reticulum (ER) (Figure 5a). No clear

229   correlation between the positioning or morphology changes of these organelles

230   and that of the mitochondrion was apparent (data not shown). We further

231   examined the apicoplast, a relict plastid that shares metabolic pathways with the

232   mitochondrion (reviewed e.g. in[18]), and for which an association with the

233   mitochondrion is well-documented[9,19]. Specifically, we examined if the apicoplast

234   is consistently found at a certain end of the sperm-like mitochondrion or at a

235   certain end or distance from the collapsed mitochondrion. We further tested

236   whether the apicoplast shape or location within the cell changes along with the

237   mitochondrial shape-change. Again, no clear correlation was apparent (data not

238   shown). However, imaging with the mitochondrial peripheral marker and a

239   parasite pellicle marker (IMC3) revealed that all three extracellular morphologies

240   show a trend of mitochondrial retraction from the tachyzoite periphery (Figure

241   5b).

242

243   The pellicle of *T. gondii* is multilayered (Figure 6a Scheme); underneath the

244   plasma membrane lays the inner membrane complex (IMC)[20]. The IMC is made

245   up of flattened membrane sacs termed alveoli, covered, on the cytoplasmic face,

246   by a network of intermediate filament-like proteins named the subpellicular

247   network[21]. The IMC encircles the parasite periphery with openings only at the

248   apical end (Figure 6a Scheme) making it a strong candidate to interact with the

249   mitochondrion and anchor its peripheral position in intracellular parasites. In

250   support of this hypothesis, super-resolution microscopy showed substantial

251   overlap in the signal from these two compartments (Figure 5b, Figure S1c

252   arrowheads), which suggests proximity of less than 200nm in the regions of

253   overlap. Immuno-electron microscopy using cryofixation and labeling the outer

254   mitochondrial membrane marker protein TgElp3[22](Figure 6b), as well as electron

255   tomography (Figure S4), detected an abundance of regions of juxtaposition of

256   mitochondrion and IMC, whereby the membranes of both organelles maintain

257   constant distance (of less than 50nm) over stretches of 100nm-1000nm. Among

258   254 random EM images of intracellular parasite sections, 39.8% presented the

259   mitochondria within these patches of tight constant distance from the IMC; 41.3%

260   presented more distant mitochondria; and 18.9% did not contain any

261   mitochondria profile in the section (Figure 6c). Within the images showing

262   stretches of mitochondrion-IMC proximity of less than 50nm, we measured an

263   average distance of 26.23 nm (+/- 12.02 nm). On the other hand, analysis of 240

264   EM images from freshly egressed extracellular parasites revealed 28.6% cases

265   of mitochondrion-IMC alignment, while 51.4% showed no tight association and

266   20% showing no mitochondria profile in the section (Figure 6c). The average

267   distance between the IMC and mitochondria at the patches of alignment seen in

268   extracellular parasites was 30.28 nm (+/- 12.33nm).

269   Finally, we tested whether the observed points of contact are stable over time by

270   performing time-lapse microscopy with frequent imaging time points (every 10

271   seconds). We could image for periods of 10-30 minutes during which we

272   observed contacts that lasted through the whole duration of the imaging (Figure

273   6d, square parentheses, Movie S7) and up to 30 minutes. Occasional transient

274   (no longer than 30 seconds) extensions towards the basal and apical end of the

275   parasite as well as inward in the direction of the nucleus are also observed

276   (Figure 6d, arrowheads).

277

278   Collectively, these data show that the mitochondrion aligns closely with the IMC,

279   and that this alignment is more extensive in intracellular parasites. We propose

280   that this intimate association contribute to the mitochondrion shape and

281   positioning seen in intracellular tachyzoites.

282

Discussion

We describe new mitochondrial behavior in the protozoan parasite *T. gondii* whereby the transition between extracellular and intracellular stages induces changes in the organelle's morphology. We identified three distinct morphological states that occur in extracellular tachyzoites and that are spotted immediately upon host cell egress; each state is observed with similar frequency in both the commonly used *in vitro* growth medium as well as a nutrient-reduced medium that more closely mimics the extracellular matrix *in vivo* (Figure 2). Likewise, the distribution of these morphologies is seen in both moving and in actively invading parasites (Figure 3). Finally, the change is reversible as collapsed mitochondria remodel into the typical lasso shape upon re-entry into the host. Taken together, these data suggest that the observed changes have physiological and functional significance.

Our observations point out a distinction between intracellular and extracellular mitochondrial morphology. The comparison between the two provided us with an opportunity to begin a dissection of the cellular features that may mediate the typical lasso shape of the mitochondria of intracellular tachyzoites. We did this by attempting to find a link between the behavior of certain cellular structures and the mitochondrial morphology. Mitochondrial morphology and localization in cells is controlled via interactions with components of the cytoskeleton and through

305   interactions with other organelles[17]. Our analyses thus focused on both these

306   factors.

307   Previous studies have shown that actin disruption results in morphological

308   defects in mitochondria in both *T. gondii*[23] and in the related parasite

309   *Plasmodium falciparum*[24]. Likewise, treatments with microtubule stability

310   inhibitors affect mitochondrial morphology ([9], Figure S3). However, the peripheral

311   distribution of the mitochondrion is still observed under both these treatments,

312   and the three morphological states seen in extracellular parasite are not

313   reproduced. Moreover, we recorded the formation of lasso-like shaped

314   mitochondria when Oryzalin treated parasites divide (Figure S3, Movie S6). We

315   conclude that while components of the cytoskeleton appear to contribute to the

316   control of mitochondrial morphology, additional factors are likely to be involved in

317   mediating the peripheral positioning of the mitochondrion of intracellular

318   tachyzoites.

319

320   Our analysis of the ER, nucleus, rhoptry and apicoplast in extracellular versus

321   intracellular tachyzoites showed no consistent change in their cellular location or

322   shape that coincided with the mitochondrial changes observed upon this

323   transition (data not shown). This suggests that the interactions that exist between

324   these organelles are not likely to contribute to the change of mitochondrial shape

325   observed in our studies.

326   The predominant feature common to the three extracellular mitochondrial

327   morphologies compared to intracellular mitochondria is a general retraction from

328 the periphery (Figure 5b), which correlates with a reduction in the number of

329 patches where the mitochondrion is closely aligned to the IMC as observed by

330 electron microscopy (Figure 6). In other eukaryotes, regions of mitochondrial

331 juxtaposition to other organelles are often attributed to the function of tether

332 complexes that enable direct transmission of signals and molecules between

333 organelles. These regions are named membrane contact sites (MCS) and have

334 been reported to be present between any two organelles that have been closely

335 studied[25,26]. The areas of apposition observed in this study between the

336 mitochondrion and the IMC have similar length, shape and distance to those

337 described for MCS, and they persist for 10-30 minutes at least (Movie S7, Figure

338 6d). We therefore hypothesize that mitochondrion-IMC MCS are one of the

339 factors responsible for the peripheral positioning and shape of mitochondria in

340 intracellular tachyzoites, and that they are reduced upon the transition to the

341 extracellular matrix. This interaction may also explain the previous observation

342 showing that upon tubulin disruption mitochondria associate with local

343 concentrations of IMC[9]. This hypothesis is also supported by findings from the

344 closely related parasite *Plasmodium falciparum*, where, in sporozoites, long

345 linker molecules that are apparently derived from the subpellicular network

346 underlying the IMC, link the IMC with mitochondria[27].

347

348 Some of the well-studied MCS involve mitochondria and include the ER–

349 Mitochondria Encounter Structure (ERMES)[28], the ER Membrane protein

350 Complex (EMC) that also functions in ER-mitochondria tethering[29], and the

351   mitochondria and plasma-membrane tethering complex Num1p/Mdm36[30] [31].

352   Mitochondrial contacts have also been observed with the yeast vacuole[32] and

353   peroxisomes[33].

354   It is hypothesized that the IMC cisternae in Apicomplexa are of ER origin[34]

355   raising the possibility that complexes that tether the ER and mitochondria in other

356   organisms may also tether the IMC and mitochondria in *T. gondii*. While no

357   homologs of the ERMES complex components are identifiable in *Toxoplasma*, a

358   set of EMC proteins homologs is present[35]*.* It has been postulated that in

359   evolutionary lineages lacking ERMES, the more evolutionarily conserved EMC

360   mediates the ER–mitochondrion tether. In humans, four other complexes were

361   proposed to mediate mitochondrial-ER tethering (reviewed in[26]). Components of

362   those complexes include the dynamin-like protein Mfn2, the mitochondrial fission

363   mediator Fis1 and the mitochondrial voltage dependent anion channel (VDAC),

364   all of which have homologs in *T. gondii* (Table S1) and are candidates to mediate

365   these potential contact sites*.* Alternatively, a novel complex that is specific to

366   these parasites or to the group of organisms containing alveoli, may tether the

367   IMC and mitochondrion. Identifying tethers and confirming the existence of

368   mitochondrial MCS in *Toxoplasma* is now a major priority in the field of organelle

369   contact sites, that otherwise focuses mainly on opisthokonts, as it would expand

370   to understand their role in divergent eukaryotes.

371

372   The possibility of mitochondrial-IMC communication raises the question of what

373   functions are supported via exchange between these two organelles. The most

374 commonly discussed functions of mitochondrial MCS are control of calcium

375 homeostasis and trafficking of lipids. The lipid composition of the IMC and

376 mitochondrion in *Toxoplasma* is not known. While lipid synthesis[36][37] and lipid-

377 dependent signalling pathways[38] that are relevant to the function of both these

378 organelles are being discovered, it is early to speculate on a potential role of

379 mitochondrial-IMC contacts in lipid exchange.

380 Previous studies provide evidence both for and against a role for mitochondrion-

381 IMC MCS in calcium homeostasis that may be similar to what is reported for

382 mitochondrial-ER exchange in other systems. Regarding the mitochondria,

383 *Toxoplasma* seems not to carry the gene encoding the mitochondrial calcium

384 uniporter (MCU)[38] that is involved in Ca^{2+} uptake in other systems. However, a

385 mitochondrial antiporter which can mediate H^+-coupled Ca^{2+} exchange has a

386 homolog in *Toxoplasma*[39]. As for the IMC, calcium storage in the *T. gondii*

387 alveolar sacs has not been examined. However the alveoli of the related

388 *Paramecium* spp act as calcium stores[40], and this is also a suggested role of the

389 IMC of the more closely related *Plasmodium* spp[41].

390 MCS that mediate calcium homeostasis expand or reduce in respond to changes

391 in calcium flux[42]. Treatments with ionomycin, which results in calcium flux in the

392 parasite cytosol, induced a significant shift in the distribution of mitochondrial

393 shapes (Figure 2c) that may be a result of change in contact site length or

394 abundance. Importantly, the observed shift is moderate. Moreover, neither

395 depletion of lipids nor a modification of the overall medium composition to mimic

396 the intracellular ion environment resulted in alteration to the mitochondrial shapes

397   observed upon egress (Figure S5). We cannot therefore conclude that there is

398   any specific strong correlation between any single condition that we have

399   analyzed and mitochondrial morphology. In this context, it is important to note

400   that as observed in other eukaryotes, the activity of multiple tethers, executing

401   various functions, may well contribute to the overall interaction between the same

402   two organelles. Our hypothesis is that the observed mitochondrial change upon

403   egress is the result of multiple changes encountered in this transition.

404   In addition to ions and lipids, other known roles of MCS that may have an effect

405   here include the control of mitochondrial inheritance after cell division, and the

406   regulation of the function of enzymes that work in trans (e.g. enzymes present on

407   one organelle but modify substrates found on the other).

408

409   Identification of the molecular machinery responsible for the establishment of the

410   patches of mitochondrion-IMC proximity would enable tackling its functional

411   significance and test the above hypotheses. Likewise, understanding whether the

412   peripheral retraction observed in extracellular parasites serves a function for

413   extracellular survival, and/or it is the result of an intracellular function that is

414   reduced upon egress, is an important step in the way of elucidating its role.

415   This work identified specific morphological states that occur to the mitochondrion

416   in extracellular parasites, which are distinct from other stress-induced

417   mitochondrial changes that have been previously described. These measures

418   can now be used to assess the outcome of genetic ablation of potential

419   mitochondrial biogenesis control mechanisms to address future questions

420   designed to better understand why these remarkable changes in the

421   mitochondrion take place.

422

423 **Methods**

424 **Parasite culture**

425 Parasites were grown in human foreskin fibroblasts (HFFs) in supplemented

426 Dulbecco's modified Eagle's medium supplemented with 2mM L-Glutamine and

427 10% Fetal Bovine Serum (we refer to this as "full medium"). To generate

428 fluorescent stable lines IMC3-YFP and TGME49_215430-tomato transgenes or

429 an endogenous YFP tagging construct for TGME49_215430 were introduced into

430 the RH based F3-line[43], following enrichment of the fluorescent population of

431 parasites stably expressing these transgenes via cell sorting using the S3e cell-

432 sorter (BioRad). Clones were isolated by limiting dilutions.

433

434 **Immunofluorescence assay**

435 All manipulations were carried out at room temperature. Intracellular parasites

436 grown in HFFs seeded on glass coverslips were fixed with 4% paraformaldehyde

437 for 20 minutes and washed in PBS. Extracellular parasites for

438 immunofluorescence assay were placed onto poly-l-lysine coated coverslips and

439 then fixed with 4% paraformaldehyde for 20 minutes and washed once with PBS.

440 Cells were permeabilized and blocked in PBS/0.02% Triton-X-100/2% BSA

441 (PBS/Triton/BSA) for 20 minutes. Slides were incubated for 60 minutes with

442 primary antibodies: anti-TGME_215430[11]; ISP1[44]; IMC1[45]; Tom40[12]; and tubulin

443 (Sigma) in PBS/Triton/BSA, washed with 3xPBS/0.02% Triton-X-100 and

444 incubated for 45 minutes with Alexa488- or Alexa594-conjugated goat anti-

445 mouse or anti-rabbit IgGs in PBS/Triton/FBS. For Mitotracker® staining HFFs

446   with parasites on glass cover slips were incubated in 300nM Mitotracker® for

447   30min at 37^0c. For Oryzalin treatment intracellular parasites were cultured in

448   presence of 2 uM Oryzalin for 18 hours after which the cells were fixed with 4%

449   PFA for 20 minutes at RT and immunofluorescence assay was carried out as

450   described above.

451

452   **Fluorescent Microscopy**

453   Micrographs were obtained using DeltaVision Core microscope

454   (AppliedPrecision) and processed using softWoRx and FIJI software. Parasites

455   with heavily distorted cell shape and fragmented mitochondria were excluded

456   from the analysis.

457   For super-resolution structural illumination microscopy (3D-SIM), stacks of 20-25

458   images were taken with increments of 0.091 µm in a Zeiss Elyra PS.1 super-

459   resolution microscope (Jena, Germany) with a 63x oil immersion objective using

460   ZEN Black software (Zeiss, Germany). Three-phase SR-SIM images were

461   reconstructed in the same software using Structural Illumination manual

462   processing tool. Maximum projection SR-SIM images and 3D models were

463   processed in Zen and FIJI softwares[46].

464

465   **Scoring Mitochondrial Morphology in Extracellular Parasites**

466   215430-YFP expressing parasites were released from HFFs by needle pass

467   (23G, Henke Sass Wolf) and filtering through 3 µm filters (VWR, 515-2036).

468   Parasites for immediate time point (Figure 2c, Figure S5) were centrifuged

469   (300RPM, 5 minutes) and fixed with 4% paraformaldehyde for 1 hour at room

470   temperature. For egress in different medium compositions intracellular parasites

471   are washed twice with the specific medium (12% full DMEM in Hanks-saline;

472   FBS free medium; K^+ buffer), parasites are then mechanically released by needle

473   pass, filter, centrifuge and fixed as above. For chemical egress (Figure 2c),

474   intracellular parasites were incubated for 10 minutes in 2 µM ionomycin (Santa

475   Cruz Biotechnology) in DMEM at 37°C prior to centrifugation and fixation. For the

476   longer time points (Figure 4b) scraped, needle passed and filtered parasites were

477   incubated in 37°C, 5% CO_2 in full medium for 6/12/24 hours before centrifugation

478   and fixation. After fixation parasites were inoculated onto poly-l-lysine (Sigma)

479   coated coverslips, allowed to adhere for 10 minutes at RT and washed once with

480   PBS. Slides were mounted in DAPI Fluoromount-G® (Cambridge Bioscience)

481   and stored at 4° C in the dark.

482   Morphologies were scored from micrographs obtained using DeltaVision Core

483   microscope (Applied Precision) with x60 objective (example in Figure 2a).

484   Parasites looking small and round and with swollen or fragmented mitochondria

485   were excluded from the analysis also in the longer incubation where their

486   numbers were high. All error bars are standard deviation (mean with SD). The

487   data for each mitochondrial shape was compared to the same mitochondrial

488   shape in the control population using paired (gliding and invasion) or unpaired

489   (egress method and media requirements) t-test. For the ionomycin experiment

490   the control is Full DMEM; for gliding and for invasion the control is the total

491   population counted from the same culture. For the 12% medium, FBS free

492   medium and K$^+$ buffer the control is full DMEM.

493

494   **Invasion assay**

495   HFFs containing 215430-YFP expressing parasites where washed to remove

496   extracellular parasites, and then scraped and needle pass (23G) to release

497   parasites. Freshly release extracellular parasites were used to infect new HFFs

498   on glass coverslips. Cells were fixed with 4% paraformaldehyde after 30 minutes

499   of incubation at 37°C. Following wash in PBS, cells were incubated in 2% BSA in

500   PBS for 20 minutes. Cells were then incubated with anti-SAG1 antibody (Abcam)

501   in 2% BSA in PBS for 1 hour and then with Alexa594 antibodies in 2% BSA in

502   PBS for 45 minutes. The slides were mounted in DAPI Fluoromount-G$^\circledR$

503   (Cambridge Bioscience) and stored at 4° C in the dark. Micrographs were

504   obtained using 3D-SIM as detailed above.

505

506   **Gliding assay**

507   HFFs containing 215430-YFP expressing parasites where washed to remove

508   extracellular parasites, and then scraped and needle pass (23G) to release

509   parasites. Freshly release extracellular parasites were inoculated on pre-coated

510   poly-l-lysine (1:10 in PBS) glass bottom dish (Cellvis) at 4°C. The live dish was

511   mounted on the imaging chamber of DeltaVision Core microscope

512   (AppliedPrecision) preheated to 37° C. Images were taken every 7 seconds for a

513   total of 10 minutes. Movie was compiled in FIJI software. For the movie used in

514 this manuscript the images were processed to account for cell drifting using the

515 ImageJ plugin, StackReg, for recursive alignment

516 (http://bigwww.epfl.ch/thevenaz/stackreg/).

517

518 **Egress assay**

519 HFFs containing 215430-YFP expressing parasites grown on glass bottom

520 dishes (Cellvis) were washed to remove extracellular parasites. The dish was

521 then mounted on the imaging chamber of DeltaVision Core microscope

522 (AppliedPrecision) preheated to 37°C for live imaging. Images were taken every

523 10 seconds for a total of 5 minutes and 2 µM ionomycin (Santa Cruz

524 Biotechnology) was added after 2-4 time points were imaged. Movie was

525 compiled in FIJI software.

526

527 **Electron microscopy**

528 RH strain *Toxoplasma* parasites expressing HA-tagged TgElp3 were prepared

529 for immunoelectron microscopy (IEM) as previously described[22]. IEM processing

530 and analysis was conducted by Wandy Beatty at Washington University, St.

531 Louis. To immunolabel sections, a 1:25 dilution of rat anti-HA (Roche) was

532 applied for 1 hour at room temperature. Samples were then incubated for another

533 hour in a 1:30 dilution of goat anti-rat antibody conjugated to 18 nm colloidal gold

534 (Jackson ImmunoResearch Laboratories) and stained with 5% uranyl acetate/2%

535 methyl cellulose. Samples were analyzed on a JEOL 1200 EX transmission

536 electron microscope (JEOL USA Inc.) with an AMT 8 megapixel digital camera

537 and AMT version 602 software (Advanced Microscopy Techniques).

538

539 **Transmission electron microscopy**

540 HFF infected cells and extracellular *T. gondii* tachyzoites were fixed with 2.5%

541 glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer. Following

542 serial washes in 0.1M phosphate buffer, the material was post-fixed in 1% OsO4

543 (vol:vol) in the same buffer for 1 hour on ice in the dark, and contrasted en bloc

544 with 0.5% aqueous uranyl acetate for 1 hour at room temperature in the dark.

545 The samples were then dehydrated in acetone ascending series and embedded

546 in epoxy resin. Ultra-thin sections (60nm) were observed in a Tecnai T20

547 transmission electron microscope (FEI, Netherlands). Images were processed

548 and analyzed in FIJI software[46]. For the mitochondrion-IMC proximity analysis,

549 254 extracellular tachyzoites were imaged and 240 of infected HFFs. For the

550 analysis, only distances less than 50 nm were considered as mitochondrion-IMC

551 contact sites. The distances between IMC and mitochondrion profiles were

552 measured in FIJI software, and data plotted in Microsft Exel software.

553

554 **Electron tomography**

555 For 3D electron tomography, 200 nm-thick sections of infected HFFs were

556 collected onto formvar-coated nickel grids. Images were recorded in tilt series

557 covering +/- 60°, at 2° increment intervals in a Jeol 2200 transmission electron

558 microscope (Jeol, Japan) operating at 200 kV equipped with a Gatan US4000

559   camera. Tilt series were aligned by cross correlation and tomogram

560   reconstruction calculated by weighted back projection using Etomo from IMOD

561   software package (Kremer et al., 1996). Segmentation and generation of the 3D

562   model were performed using 3dmod program of the same software package

563

564

565 References
566

567 1   Collins, T. J. and Bootman, M. D., Mitochondria are morphologically
568   heterogeneous within cells. *J Exp Biol* **206** (Pt 12), 1993 (2003).
569 2   Kasahara, A. et al., Mitochondrial fusion directs cardiomyocyte differentiation
570   via calcineurin and Notch signaling. *Science* **342** (6159), 734 (2013).
571 3   Gorsich, S. W. and Shaw, J. M., Importance of mitochondrial dynamics during
572   meiosis and sporulation. *Mol Biol Cell* **15** (10), 4369 (2004).
573 4   Priest, J. W. and Hajduk, S. L., Developmental regulation of mitochondrial
574   biogenesis in *Trypanosoma brucei. J Bioenerg Biomembr* **26** (2), 179 (1994).
575 5   Schneider, A., Unique aspects of mitochondrial biogenesis in
576   trypanosomatids. *Int J Parasitol* **31** (13), 1403 (2001).
577 6   Schnaufer, A., Clark-Walker, G. D., Steinberg, A. G., and Stuart, K., The F1-ATP
578   synthase complex in bloodstream stage trypanosomes has an unusual and
579   essential function. *EMBO J* **24** (23), 4029 (2005).
580 7   Melo, E. J., Attias, M., and De Souza, W., The single mitochondrion of
581   tachyzoites of *Toxoplasma gondii. J Struct Biol* **130** (1), 27 (2000).
582 8   Seeber, F., Ferguson, D. J., and Gross, U., *Toxoplasma gondii*: a
583   paraformaldehyde-insensitive diaphorase activity acts as a specific
584   histochemical marker for the single mitochondrion. *Exp Parasitol* **89** (1), 137
585   (1998).
586 9   Nishi, M., Hu, K., Murray, J. M., and Roos, D. S., Organellar dynamics during the
587   cell cycle of *Toxoplasma gondii. J Cell Sci* **121** (Pt 9), 1559 (2008).
588 10   Konradt, C. et al., Endothelial cells are a replicative niche for entry of
589   *Toxoplasma gondii* to the central nervous system. *Nat Microbiol* **1**, 16001
590   (2016).
591 11   MacRae, J. I. et al., Mitochondrial metabolism of glucose and glutamine is
592   required for intracellular growth of *Toxoplasma gondii. Cell Host Microbe* **12**
593   (5), 682 (2012).
594 12   van Dooren, G. G., Yeoh, L. M., Striepen, B., and McFadden, G. I., The Import of
595   Proteins into the Mitochondrion of *Toxoplasma gondii. J Biol Chem* **291** (37),
596   19335 (2016).
597 13   Wang, Y., Weiss, L. M., and Orlofsky, A., Host cell autophagy is induced by
598   *Toxoplasma gondii* and contributes to parasite growth. *J Biol Chem* **284** (3),
599   1694 (2009).
600 14   Ghosh, D., Walton, J. L., Roepe, P. D., and Sinai, A. P., Autophagy is a cell death
601   mechanism in *Toxoplasma gondii. Cell Microbiol* **14** (4), 589 (2012).
602 15   Lavine, M. D. and Arrizabalaga, G., Analysis of monensin sensitivity in
603   *Toxoplasma gondii* reveals autophagy as a mechanism for drug induced
604   death. *PLoS One* **7** (7), e42107 (2012).
605 16   Sheiner, L., Dowse, T. J., and Soldati-Favre, D., Identification of trafficking
606   determinants for polytopic rhomboid proteases in *Toxoplasma gondii. Traffic*
607   **9** (5), 665 (2008).
608 17   Labbe, K., Murley, A., and Nunnari, J., Determinants and functions of
609   mitochondrial behavior. *Annu Rev Cell Dev Biol* **30**, 357 (2014).

610   18   Sheiner, L., Vaidya, A. B., and McFadden, G. I., The metabolic roles of the
611   endosymbiotic organelles of Toxoplasma and Plasmodium spp. *Curr Opin*
612   *Microbiol* **16** (4), 452 (2013).
613   19   Kobayashi, T. et al., Mitochondria and apicoplast of *Plasmodium falciparum*:
614   behaviour on subcellular fractionation and the implication. *Mitochondrion* **7**
615   (1-2), 125 (2007).
616   20   Harding, C. R. and Meissner, M., The inner membrane complex through
617   development of *Toxoplasma gondii* and Plasmodium. *Cell Microbiol* **16** (5),
618   632 (2014).
619   21   Mann, T. and Beckers, C., Characterization of the subpellicular network, a
620   filamentous membrane skeletal component in the parasite *Toxoplasma*
621   *gondii. Mol Biochem Parasitol* **115** (2), 257 (2001).
622   22   Stilger, K. L. and Sullivan, W. J., Jr., Elongator protein 3 (Elp3) lysine
623   acetyltransferase is a tail-anchored mitochondrial protein in *Toxoplasma*
624   *gondii. J Biol Chem* **288** (35), 25318 (2013).
625   23   Shaw, M. K., Compton, H. L., Roos, D. S., and Tilney, L. G., Microtubules, but not
626   actin filaments, drive daughter cell budding and cell division in *Toxoplasma*
627   *gondii. J Cell Sci* **113 (Pt 7)**, 1241 (2000).
628   24   Hliscs, M. et al., Organization and function of an actin cytoskeleton in
629   *Plasmodium falciparum* gametocytes. *Cell Microbiol* **17** (2), 207 (2015).
630   25   Elbaz, Y. and Schuldiner, M., Staying in touch: the molecular era of organelle
631   contact sites. *Trends Biochem Sci* **36** (11), 616 (2011).
632   26   Prinz, W. A., Bridging the gap: membrane contact sites in signaling,
633   metabolism, and organelle dynamics. *J Cell Biol* **205** (6), 759 (2014).
634   27   Kudryashev, M. et al., Positioning of large organelles by a membrane-
635   associated cytoskeleton in Plasmodium sporozoites. *Cell Microbiol* **12** (3),
636   362 (2010).
637   28   Kornmann, B. et al., An ER-mitochondria tethering complex revealed by a
638   synthetic biology screen. *Science* **325** (5939), 477 (2009).
639   29   Lahiri, S. et al., A conserved endoplasmic reticulum membrane protein
640   complex (EMC) facilitates phospholipid transfer from the ER to
641   mitochondria. *PLoS Biol* **12** (10), e1001969 (2014).
642   30   Klecker, T., Scholz, D., Fortsch, J., and Westermann, B., The yeast cell cortical
643   protein Num1 integrates mitochondrial dynamics into cellular architecture. *J*
644   *Cell Sci* **126** (Pt 13), 2924 (2013).
645   31   Lackner, L. L. et al., Endoplasmic reticulum-associated mitochondria-cortex
646   tether functions in the distribution and inheritance of mitochondria. *Proc*
647   *Natl Acad Sci U S A* **110** (6), E458 (2013).
648   32   Elbaz-Alon, Y. et al., A dynamic interface between vacuoles and mitochondria
649   in yeast. *Dev Cell* **30** (1), 95 (2014).
650   33   Cohen, Y. et al., Peroxisomes are juxtaposed to strategic sites on
651   mitochondria. *Mol Biosyst* **10** (7), 1742 (2014).
652   34   de Melo, E. J. and de Souza, W., A cytochemistry study of the inner membrane
653   complex of the pellicle of tachyzoites of *Toxoplasma gondii. Parasitol Res* **83**
654   (3), 252 (1997).

655   35   Wideman, J. G. and Munoz-Gomez, S. A., The evolution of ERMIONE in
656   mitochondrial biogenesis and lipid homeostasis: An evolutionary view from
657   comparative cell biology. *Biochim Biophys Acta* (2016).
658   36   Bisanz, C. et al., *Toxoplasma gondii* acyl-lipid metabolism: de novo synthesis
659   from apicoplast-generated fatty acids versus scavenging of host cell
660   precursors. *Biochem J* **394** (Pt 1), 197 (2006).
661   37   Hartmann, A. et al., Phosphatidylethanolamine synthesis in the parasite
662   mitochondrion is required for efficient growth but dispensable for survival of
663   *Toxoplasma gondii. J Biol Chem* **289** (10), 6809 (2014).
664   38   Bullen, H. E. et al., Phosphatidic Acid-Mediated Signaling Regulates
665   Microneme Secretion in Toxoplasma. *Cell Host Microbe* **19** (3), 349 (2016).
666   39   Rotmann, A. et al., PfCHA is a mitochondrial divalent cation/H+ antiporter in
667   *Plasmodium falciparum. Mol Microbiol* **76** (6), 1591 (2010).
668   40   Ladenburger, E. M., Sehring, I. M., Korn, I., and Plattner, H., Novel types of
669   Ca2+ release channels participate in the secretory cycle of Paramecium cells.
670   *Mol Cell Biol* **29** (13), 3605 (2009).
671   41   Holder, A. A., Mohd Ridzuan, M. A., and Green, J. L., Calcium dependent
672   protein kinase 1 and calcium fluxes in the malaria parasite. *Microbes Infect*
673   **14** (10), 825 (2012).
674   42   Idevall-Hagren, O., Lu, A., Xie, B., and De Camilli, P., Triggered Ca2+ influx is
675   required for extended synaptotagmin 1-induced ER-plasma membrane
676   tethering. *EMBO J* **34** (17), 2291 (2010).
677   43   Sheiner, L. et al., A systematic screen to discover and analyze apicoplast
678   proteins identifies a conserved and essential protein import factor. *PLoS*
679   *Pathog* **7** (12), e1002392 (2011).
680   44   Beck, J. R. et al., A novel family of Toxoplasma IMC proteins displays a
681   hierarchical organization and functions in coordinating parasite division.
682   *PLoS Pathog* **6** (9), e1001094 (2010).
683   45   Wichroski, M. J. et al., Clostridium septicum alpha-toxin is active against the
684   parasitic protozoan *Toxoplasma gondii* and targets members of the SAG
685   family of glycosylphosphatidylinositol-anchored surface proteins. *Infect*
686   *Immun* **70** (8), 4353 (2002).
687   46   Schindelin, J. et al., Fiji: an open-source platform for biological-image
688   analysis. *Nat Methods* **9** (7), 676 (2012).
689   47   Sadak, A., Taghy, Z., Fortier, B., and Dubremetz, J. F., Characterization of a
690   family of rhoptry proteins of *Toxoplasma gondii. Mol Biochem Parasitol* **29** (2-
691   3), 203 (1988).
692   48   Agrawal, S., van Dooren, G. G., Beatty, W. L., and Striepen, B., Genetic evidence
693   that an endosymbiont-derived endoplasmic reticulum-associated protein
694   degradation (ERAD) system functions in import of apicoplast proteins. *J Biol*
695   *Chem* **284** (48), 33683 (2009).
696
697
698
699
700

717 **Author Contributions**
718
719 L.S. conceived and designed the study, interpreted the data and wrote the paper.
720 L.L. performed and analyzed the transmission electron microscopy and
721 contributed to writing the manuscript. J.O. optimized live microscopy conditions,
722 developed morphology scoring system, designed and implemented all other
723 experimental details and contributed to writing the manuscript. KLS generated
724 TgElp3 expressing parasites and provided immune-EM data. WJS designed
725 TgElp3 studies and contributed to writing the manuscript.
726
727 **Competing Financial Interest Declaration**
728
729 The authors read and understood NPG policy on competing financial interest
730 (http://www.nature.com/authors/policies/competing.html) and we declare no
731 competing financial interests.
732

Figure 1 A new peripheral marker defines additional mitochondrial structures to a matrix marker. Immunofluorescence micrograph of the mitochondria of two intracellular *T. gondii* tachyzoite expressing 215430-YFP (green) and stained with anti-Tom40 antibody **(a)** or Mitotracker® **(b).** All panels show Z projection. **(c,d)** Immunofluorescence micrograph of the mitochondria of two intracellular *T. gondii* tachyzoite expressing HSP60-RFP (magenta) and 215430-YFP (green). All panels show Z projection. Arrowhead in **(c)** highlight an example of 215430 marking additional structures to the matrix marker. Arrowheads in **(d)** point to places where the matrix signal break and the 215430 signal is continues. Bars 2μm.

Figure 2 Mitochondrial morphology of *T. gondii* tachyzoites changes upon host cell egress. **(a)** Fluorescence micrograph of intracellular (In) and extracellular (out) populations of *T. gondii* tachyzoites taken utilizing the signal from 215430-YFP (green in the merge with the brightfield, top panels, and in the bottom panels). Bars 5μm. **(b)** Three representative images of each of the observed shapes of mitochondria in extracellular parasites and their classification. Each example shows the fluorescent signal image on the left and the merge of fluorescence and brightfield on the right. The color-coding of the frame (lasso – green; sperm-like – orange; collapsed – red) is maintained throughout all the figures. **(c)** Proportions of the morphologies scored in intracellular parasites (Intracellular, 776 parasites, over 6 independent experiments); in parasites mechanically released from host cell into full growth medium (Full, 864 parasites, over 9 independent experiments) or into diluted growth medium (12%, 653 parasites, over 2 independent experiments) immediately after release; and in parasites induced to egress by 2μM ionomycin (Ionomycin, 1177 parasites, over 6 independent experiments) immediately after release. Error bars are standard deviation. **(d)** Super-resolution microscopy images of the mitochondrial lasso morphology in intracellular (left) and the three main mitochondrial morphologies observed in extracellular: lasso, sperm-like and collapsed (right) shown as projection of all Z stacks (top) and as 3D reconstruction (bottom). 215430 - green. DAPI - blue. Bar 2μm.

Figure 3. The change in the mitochondrial morphology of extracellular tachyzoites is seen in motile and in actively invading parasites. (a) A scheme depicting the main stages of the tachyzoite lytic cycle. **(b)** Snapshots from time-lapse microscopy of gliding parasites with different mitochondrial morphologies (Movie S3). Each parasite is numbered to enable following its trajectory. Time points (min:sec) are shown in each of the images. Bar - 10μm. **(c)** Distribution of lasso, sperm-like and collapsed morphologies in motile parasites (148 over 4 independent experiments), compared to the distribution in the total extracellular population (e.g. motile + non-motile, 171 parasites over the same 4 experiments), and to the distribution scored in intracellular parasites in the experiment presented in Figure 2c. **(d)** Fluorescence images of invading parasites with lasso (left), sperm-like (middle) and collapsed (right) mitochondria. Snapshots from movies S4.1/2/3. TGME49_ 215430 (green). SAG1 (magenta)

779 staining was obtained without permeabilisation to visualize parasite that are half
780 way into the host (i.e. only the extracellular part of the parasites is accessible to
781 SAG1 antibody) as depicted in the scheme on the left. Bars - 1μm. **(e)**
782 Distribution of mitochondrial morphologies in invading parasites (223 over 3
783 independent experiments) that were allowed to invade immediately after
784 mechanical release from host cells, compared to the population of extracellular
785 parasites (621 over 3 independent experiments) obtained from the same
786 preparation of egressed parasites, and to the distribution scored in intracellular
787 parasites in the experiment presented in Figure 2c. Error bars in (c) and (e) are
788 standard deviation.
789
790 **Figure 4. The observed morphological change is reversible upon host cell**
791 **invasion and directional. (a)** Snapshots from time-lapse microscopy of a
792 parasite with collapsed mitochondrion after invasion and until completion of the
793 first round of division (from Movie S5). 215430-YFP – green. IMC3 – magenta.
794 Bar - 2μm. **(b)** Proportions of morphologies scored in parasites mechanically
795 released from host cell after 6/12/24 hours of extracellular incubation (404, 930,
796 419 parasites were scored over 3,9,3 independent experiments respectively).
797 These are compared to scores obtained immediately after release or in
798 intracellular parasites from the experiment shown in Figure 2c. Error bars are
799 standard deviation. **(c)** Proportion of basal facing and apical facing "tail" in
800 sperm-like mitochondria (190 parasites over 3 independent experiments, all
801 counted after 6 hours of extracellular incubation, the time point with most sperm-
802 like morphologies). The immunofluorescence on the right demonstrates
803 examples of basal and apical facing tails. TGME49_ 215430 – green. ISP1 –
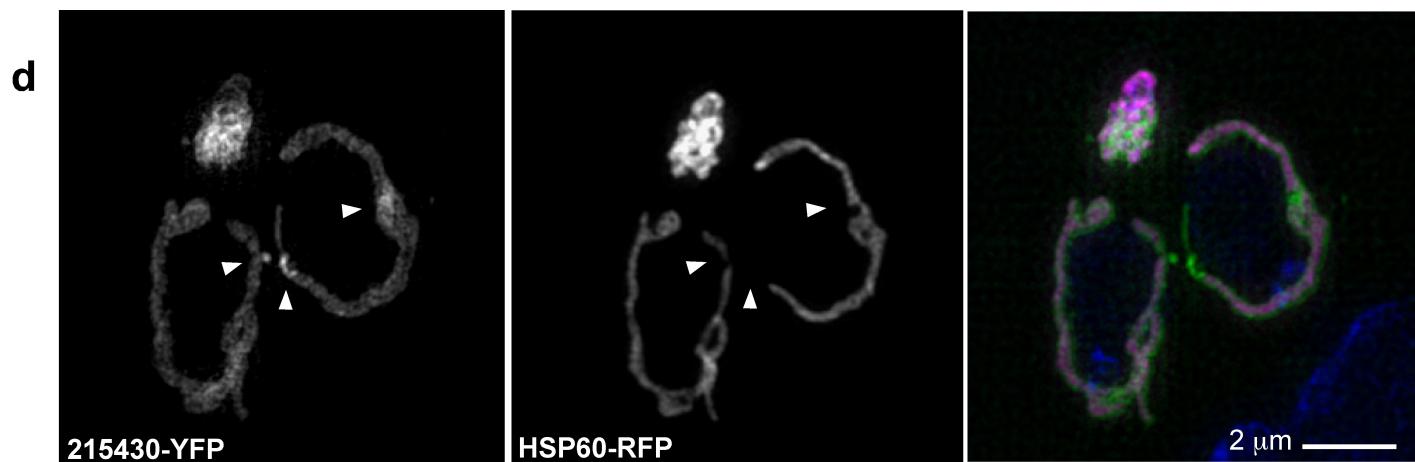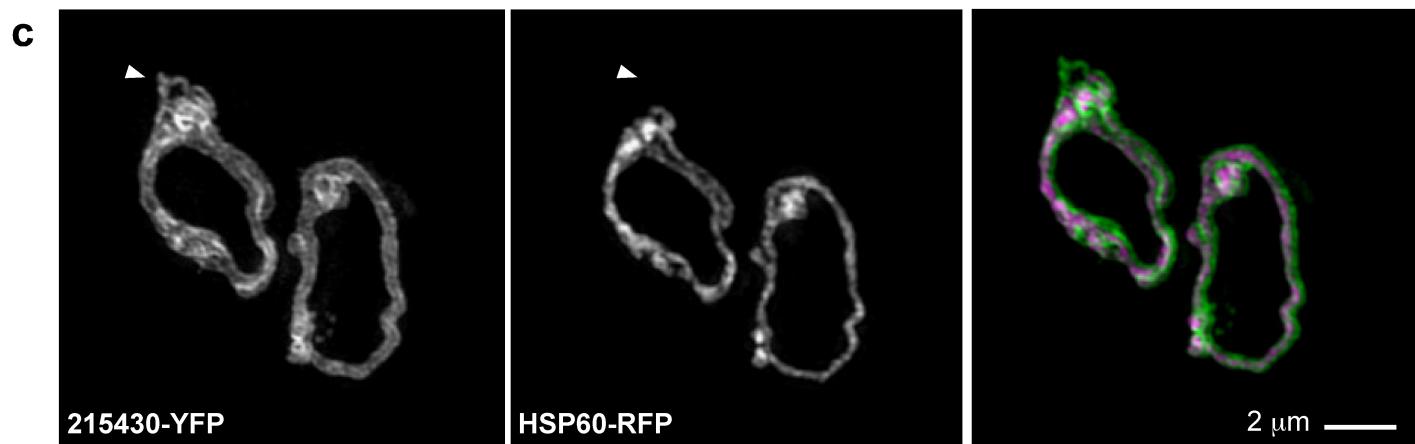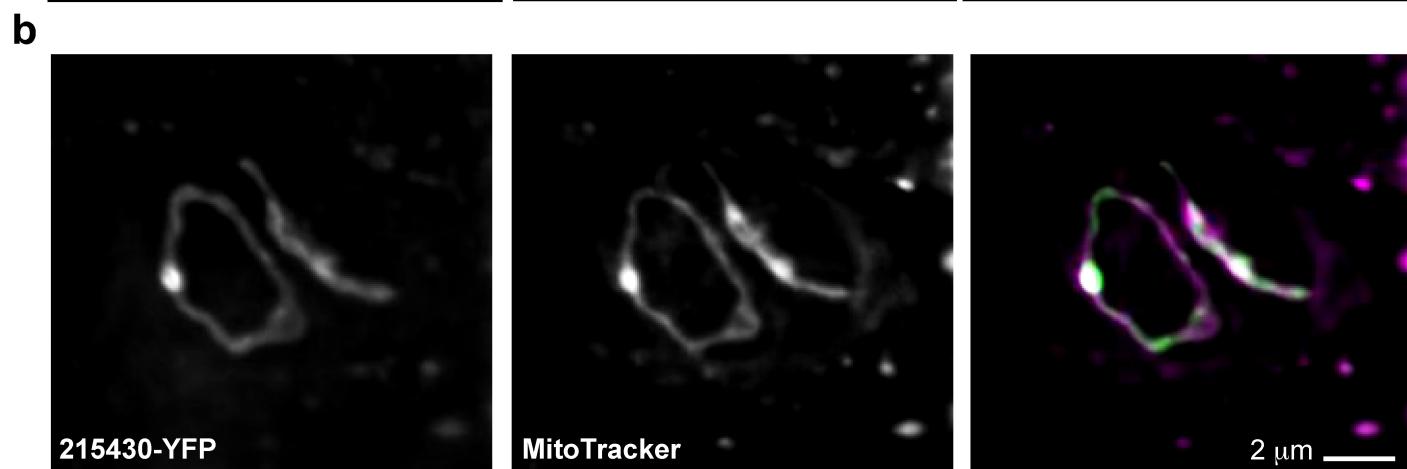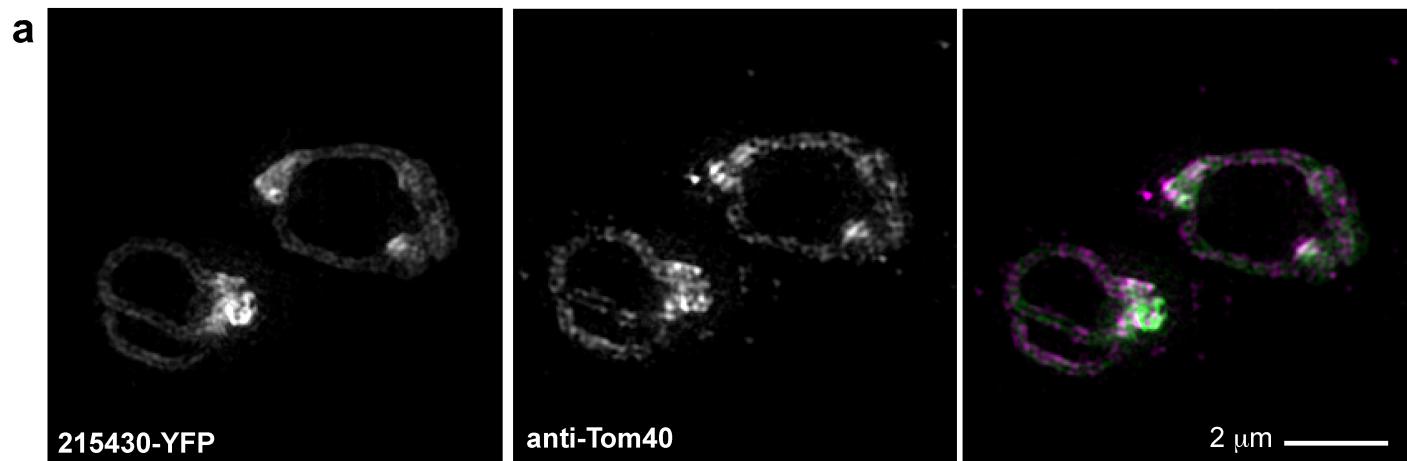804 magenta. DAPI - blue. Bar - 1μm.
805
806
807 **Figure 5. Analysis of morphology and positioning of other organelles in**
808 **relation to the observed mitochondrial change reveals retraction from the**
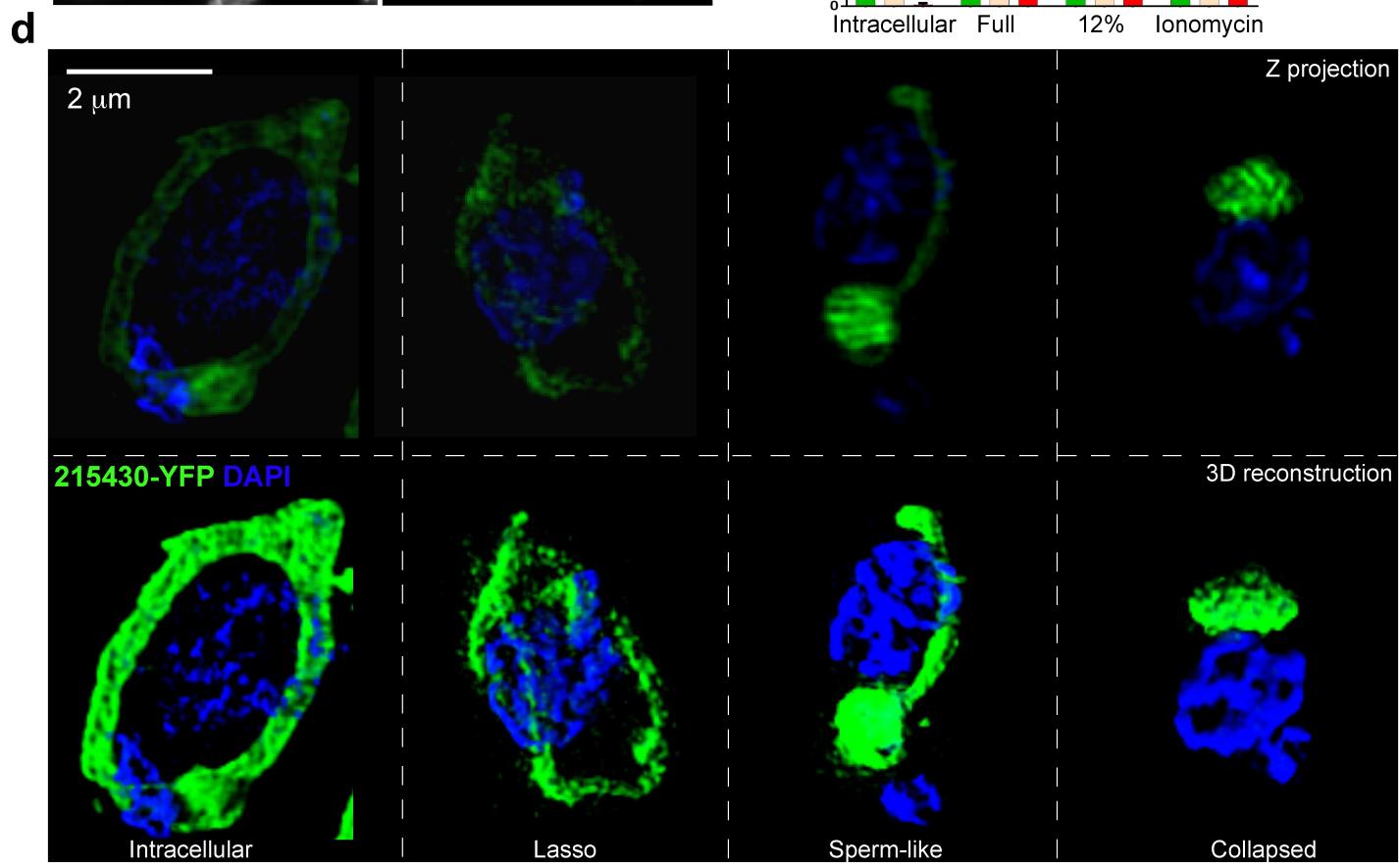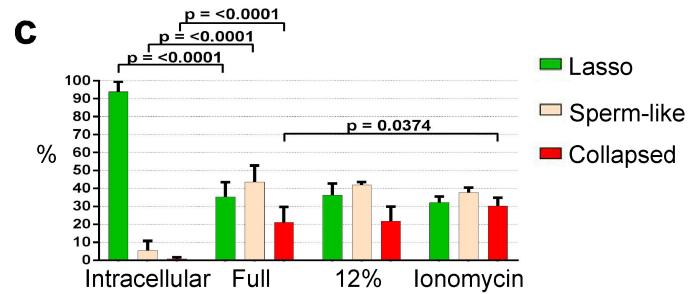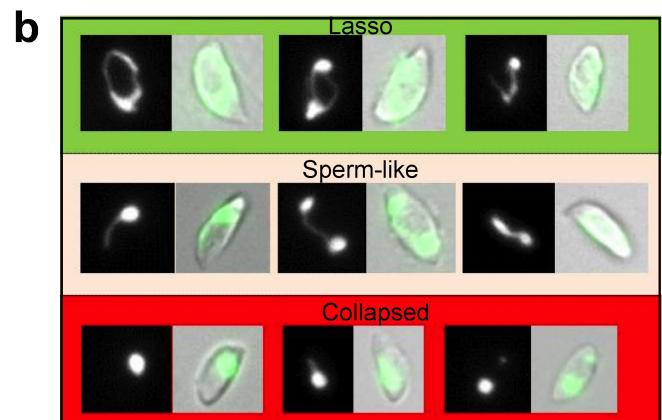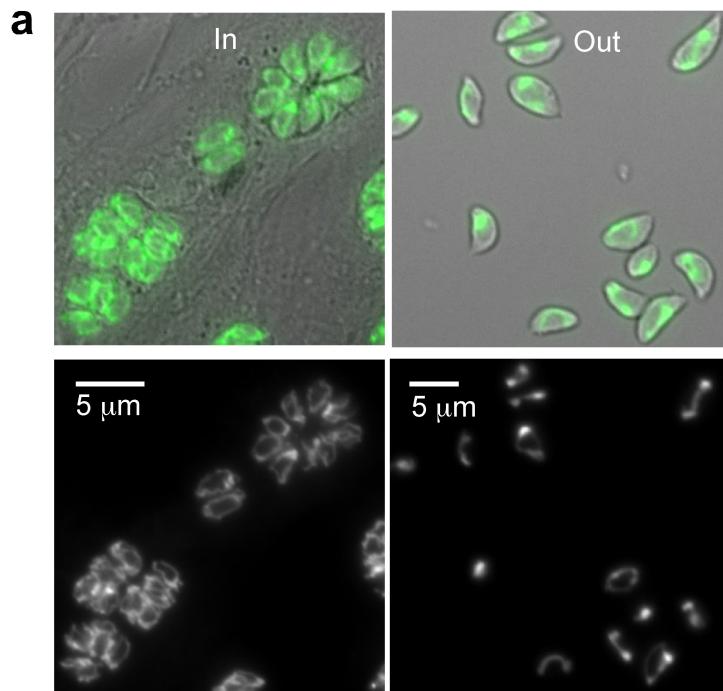809 **parasite periphery as the main consistent feature of this change. (a)**
810 Randomly selected micrographs of the co-staining of mitochondrion and (i)
811 nucleus (ii) apicoplast (iii) rhoptries (vi) ER in intracellular (in) and extracellular
812 (out) parasites. **(b)** Representative micrographs of the co-staining of
813 mitochondrion and IMC in intracellular (in) and extracellular (out) parasites. Bars
814 - 2μm. TGME49_ 215430 - green. Anti-Rop4 antibody (Rop2,4 - T34A7[47]),/Der-
815 GFP[48]/DAPI/anti-CPN60 – magenta.
816
817
818 **Figure 6. Mitochondrial peripheral retraction is linked to reduction in IMC**
819 **tight-proximity zones. (a)** Schematic depiction of two tachyzoites showing their
820 mitochondria (green), plasma membrane (grey), microtubules (dark purple), inner
821 membrane complex (magenta) and sub pellicular microtubules (fuzzy grey). **(b)**
822 Cryo-immuno-EM using parasites expressing the mitochondrial associated
823 TgElp3-HA[22](labeled with gold beads). White arrows show IMC. Black arrows
824 show the plasma membrane. **(c)** Frequency of sections showing patches of
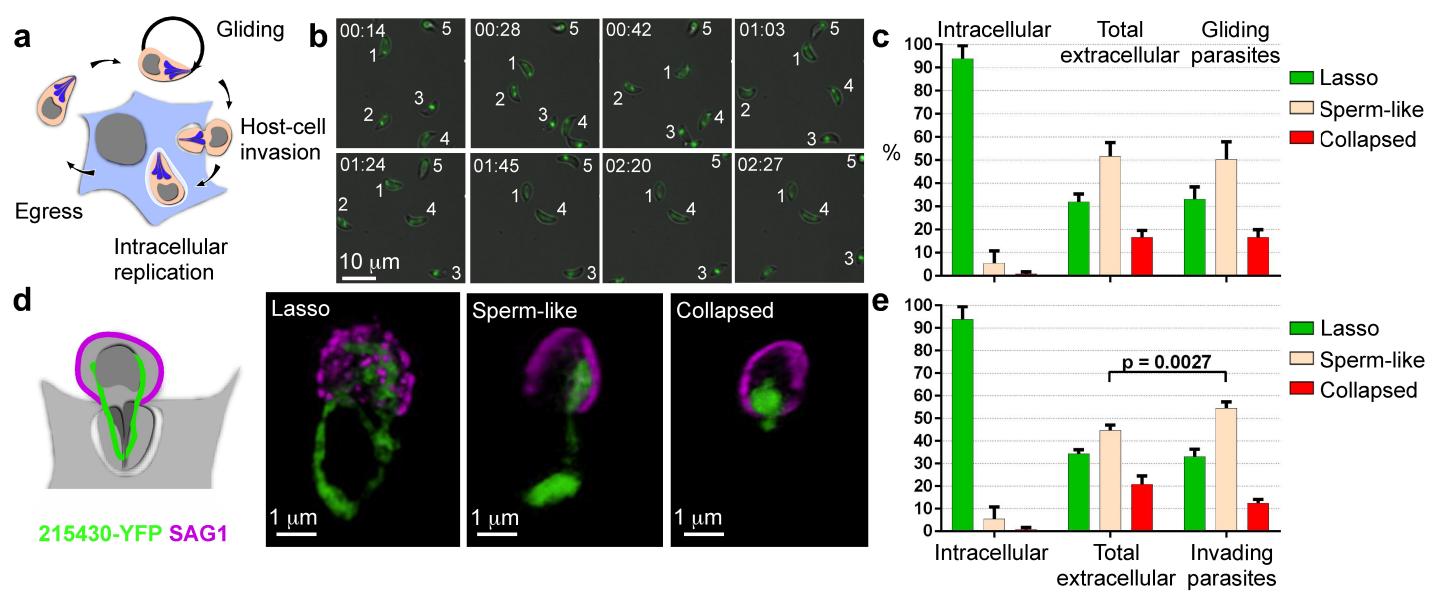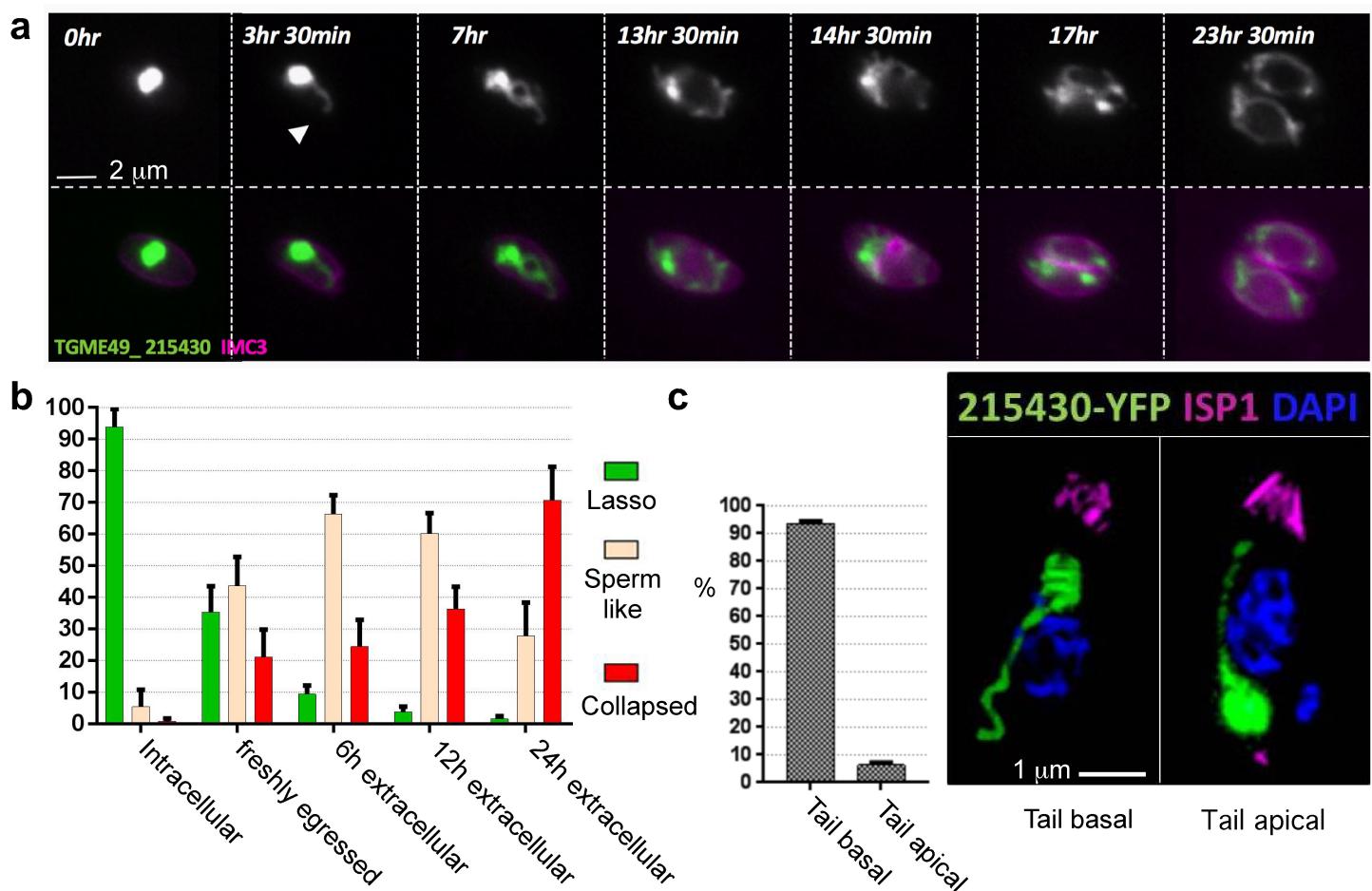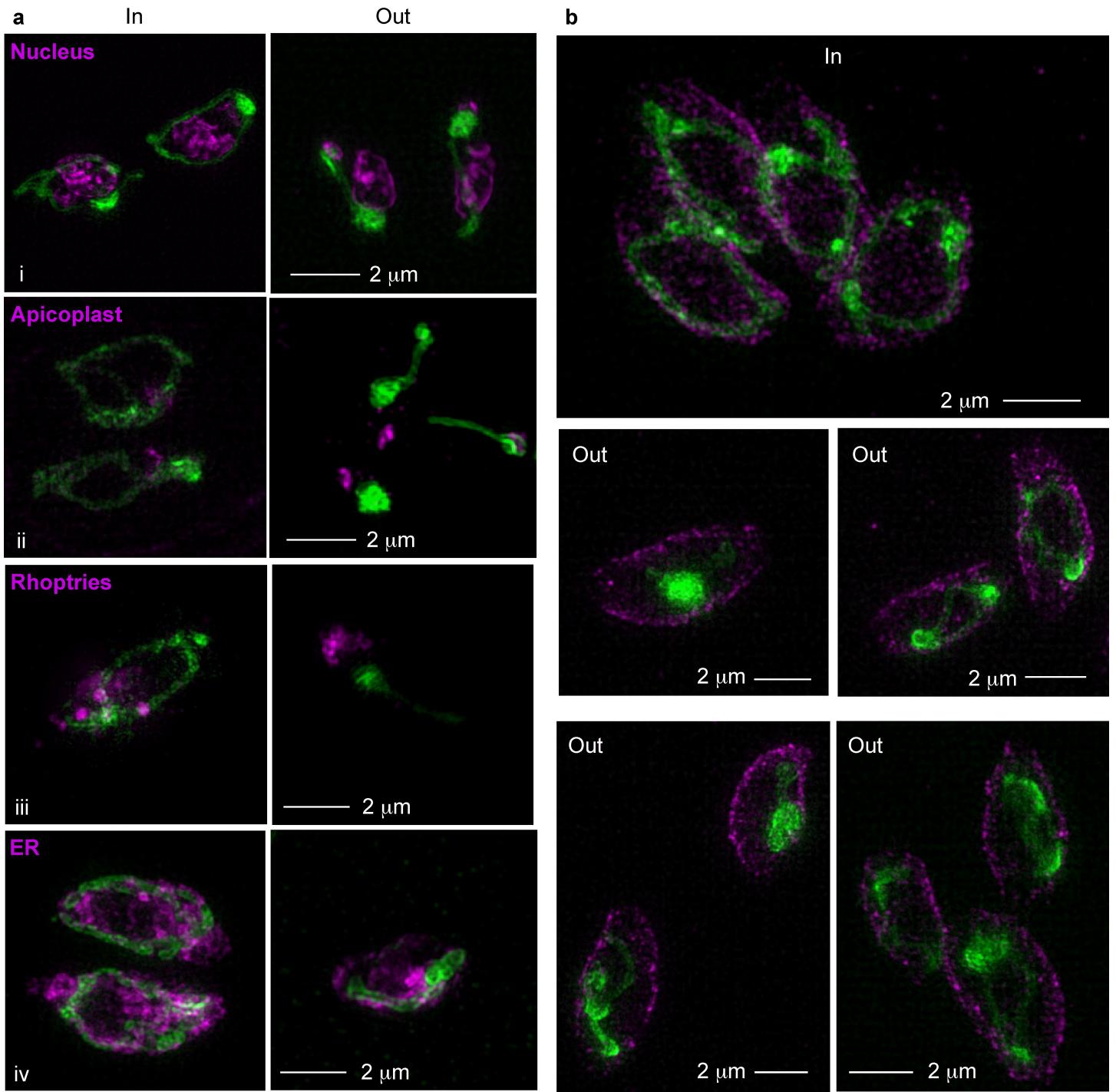
825   mitochondrial–IMC proximity of <50nm or >50nm distance and of section not
826   showing mitochondrial profile, among EM sections of intracellular and
827   extracellular parasites. **(d)** Snapshots from the time-lapse microscopy shown in
828   Movie S7. Merge panel shows TGME49_ 215430 in green and IMC3 in magenta,
829   and each channel is also shown separately (IMC in the middle and TGME49_
830   215430 at the bottom). Square parentheses highlight regions of close IMC-
831   mitochondrion contact that are stable over the period of live imaging. Arrowheads
832   mark region of transient extensions from the mitochondrial tubule.
833

a

215430-YFP | anti-Tom40 | 2 µm

b

215430-YFP | MitoTracker | 2 µm

c

215430-YFP | HSP60-RFP | 2 µm

d

215430-YFP | HSP60-RFP | 2 µm

a

In | Out

5 μm | 5 μm

b

Lasso

Sperm-like

Collapsed

c

p = <0.0001
p = <0.0001
p = <0.0001
p = 0.0374

Lasso
Sperm-like
Collapsed

%

100
90
80
70
60
50
40
30
20
10
0

Intracellular | Full | 12% | Ionomycin

d

Z projection

2 μm

215430-YFP DAPI

3D reconstruction

Intracellular | Lasso | Sperm-like | Collapsed

a Gliding / Host-cell invasion / Egress / Intracellular replication

b 00:14 00:28 00:42 01:03 01:24 01:45 02:20 02:27 10 μm

c Intracellular / Total extracellular / Gliding parasites
Lasso / Sperm-like / Collapsed

d 215430-YFP SAG1 / Lasso / Sperm-like / Collapsed / 1 μm

e Intracellular / Total extracellular / Invading parasites
p = 0.0027
Lasso / Sperm-like / Collapsed

a

0hr · 3hr 30min · 7hr · 13hr 30min · 14hr 30min · 17hr · 23hr 30min

2 μm

TGME49_ 215430 IMC3

b

Lasso
Sperm like
Collapsed

Intracellular · freshly egressed · 6h extracellular · 12h extracellular · 24h extracellular

c

215430-YFP ISP1 DAPI

%

Tail basal · Tail apical

1 μm

Tail basal · Tail apical

a

In | Out

i **Nucleus**

2 μm

ii **Apicoplast**

2 μm

iii **Rhoptries**

2 μm

iv **ER**

2 μm

b

In

2 μm

Out

2 μm

Out

2 μm

Out

2 μm

Out

2 μm

a

Plasma membrane
Inner membrane complex
Sub pellicular netwrok
Microtubules
Mitochondrion

b

500nm

250nm

100nm

100nm

c

Intracellular Extracellular

%

IMC proximity <50nm
IMC proximity >50nm
No mito-chondrion in image

d

00:00 02:40 05:10 07:40 11:10 14:00

2 µm

IMC3

215430-YFP